COMPARATIVE HISTOCHEMICAL OBSERVATIONS ON WOUND HEALING IN ADULT RATS AND CULTURED ADULT HUMAN EPITHELIUM

II. Ribonucleic Acid and Thymonucleic Acid*†

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During wound healing epidermis first undergoes a phase of cellular migration followed by one of growth. In rats, the latter phase is characterized by an increased concentration of cytoplasmic ribonucleic acid. This is restricted to those cells where mitosis occurs, namely in the basal and lower stratum spinosum layer. A similar response is found in cultured human epithelium also being limited to those layers where proliferation occurs.

During culture, explants frequently reveal areas of separation at the dermo-epidermal junction. Epithelium overlying these areas is morphologically abnormal with degeneration in the lower layers and preservation of a "basal cell appearance" in the more superficial layers.

A close similarity exists between epithelium migrating over denuded burn surfaces and the outwandering cells found in culture. In both cases the initial migration is followed by intense proliferation just within the borders either of the surrounding normal skin (in burns) or of the explant (in culture). Mitosis occurs to a lesser extent in the outwandering epithelium during culture but a large percentage of these are abnormal. Thymonucleic acid concentration of the nuclei either in burns or culture does not appear to vary appreciably.

The ribonucleic and thymonucleic acid metabolism of epithelial cells during *in vivo* wound healing and *in vitro* cultures appears identical.

Similarities between reepithelialization of adult rat burns and proliferation of adult human skin in culture are of a chemical as well as morphological nature. Glycogen metabolism under these diverse conditions bears striking resemblances (Washburn '54). It is conceivable that such similarities also extend to protein metabolism. Epithelium either in healing wounds or in culture possesses several common physiologic activities. These consist of rapid cellular migration and proliferation without keratinization. It has now been amply demonstrated in a host of tissues that a high ribonucleic acid (R.N.A.) content is characteristic of cells in which protein synthesis is vigorous either for purposes of growth or secretion. This concept relating R.N.A. to protein synthesis was arrived at by two entirely different technics. Caspersson (9) employed the ultra-violet absorption properties of R.N.A. while Brachet (6) utilized its basophilic staining

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nature in conjunction with ribonuclease digested controls. In 1944 Brachet and Jeener concluded that the cytoplasmic proteins are synthesized by microsomes, —ultracentrifugable cytoplasmic particles to which the majority of R.N.A. is bound.

With the establishment of a histochemical technic for the identification of R.N.A., observations concerning its location in skin were made. Brachet (6) observed a decreasing gradient from the basal cell layer to the stratum corneum. This was extended by Nolte (28) who using the human toe described the relative absence of R.N.A. in the outer layers of the stratum spinosum as compared to its inner layers. Observations on mouse skin (Hardy (20)) substantiate these findings. It is interesting to note that the former site, in contrast to the latter, reveals little cell division. These observations concerning the presence of R.N.A. in skin have also been confirmed with biochemical assays in terms of nucleic acid phosphorus (Davidson and Waymouth (13)).

It would be expected therefore that there would be an increase in R.N.A. in proliferating epithelium following wounding. This has been shown to be the case in both the mouse (Clement (10)) and the guinea pig (Firket (18)). These authors observed that the increase occurred only at those periods when mitosis was active and not during the migrating phase of epithelialization. If cultured epithelium proliferates *in vitro* in a normal manner, a similar increase in cytoplasmic R.N.A. content would be expected.

Thymonucleic acid (D.N.A.) found only in the nucleus, has been studied histochemically since Feulgen and Rossenbeck (17) observed that mild acid hydrolysis released aldehyde groups which coloured with Schiff's reagent. Like R.N.A. it is also increased in actively multiplying cells. Present concepts are that this increase is indirectly related to protein synthesis, the nucleus controlling the number of microsomes which in turn are the agents of protein synthesis, probably by autoduplication (Brachet (8)). The nuclei of skin epithelium reveal that thymonucleic acid is most concentrated in the basal cell and lower stratum spinosum layers (Hardy (20)). The more superficial mitotic layers contain relatively less. Skin proliferating in excess of a normal rate contains greater quantities of D.N.A. Stowell has measured quantitatively by means of the photometric method the amount of D.N.A. in methylcholanthrene induced mouse skin cancer (33) and in human epidermoid carcinoma as well as hyperplastic lesions (34) (36). In all three cases the D.N.A. content was significantly elevated per unit volume and per cell. More recently Bunting et al. (5) observed the same phenomenon visually in a virus induced papilloma. The amounts present in nuclei of epithelial cells during wound healing or cultured *in vitro* have not been subjected to study.

As both ribonucleic acid and thymonucleic acid are closly related to growth and proliferation, it was decided to re-evaluate and compare in this light the problems of:

1. Reepithelialization of adult rat wounds *in vivo*.
2. Proliferation of adult human skin cultured *in vitro*.

MATERIALS AND METHODS

The technics for obtaining uniform rat burns and the methods of culture used for human skin were discussed in the first paper of this series and will not be repeated.

Histochemical Technics

1. *Fixatives:* For ribonucleic acid two solutions were employed. Those stained by the Unna-Brachet method were first fixed for 14–18 hours, in 10% buffered Formal (pH 7) at room temperature. Those stained by methylene blue were fixed for 24 hours at room temperature in a saturated aqueous solution of mercuric chloride 50 parts and 95% alcohol 50 parts.

For thymonucleic acid all fixations were carried out during 24 hours in zenker-acetic acid.

2. *Staining:* Ribonucleic acid was detected by two methods based on its basophilic staining characteristics. The pyronine G-methyl green stain of Brachet '42 was used with slight modification, the solution being placed in an acetate buffer system at pH 4.8 (Trevan and Sharrock '51). Other sections were progressively stained in a 0.003% solution of methylene blue chloride buffered to pH 7.6 with KH_2PO_4.

In all cases an equal number of control sections were digested for 2–4 hours at 60°C. in a 0.033% aqueous solution of ribonuclease (Worthington).

The Feulgen reaction for thymonucleic acid was carried out by hydrolysing sections in normal HCL. at 60°C. for 30–60 minutes prior to immersion in Schiff's reagent, 30 min. Lison (26) and Stowell (35). Control sections were similarly treated excepting that water was substituted for HCL.

RESULTS

Rat Burns

The basic dye which stains ribonucleic acid because of its acidity is observed to be concentrated in the basal and lower stratum spinosum layers of normal epidermis; in those layers directly above, the uptake is considerably less. This basophilia is removed by the activity of ribonuclease, thus confirming that its presence is due to R.N.A. The keratinized stratum corneum however also stains very heavily with basic dyes but ribonuclease has no effect upon it. This is good evidence that the dye uptake in the stratum corneum is due to some basophilic substance other than R.N.A. Normal skin presents an R.N.A. gradient extending from the basal layer to the stratum lucidum with decreasing intensity.

To study the relative skin concentration of R.N.A. in wound healing 28 rat burns were employed. All were sectioned at 7 micra and visual differences observed in the concentration of the basic dye. During the first 2 days following burning, the epithelium of the wound edge becomes thickened, with an increase in the number of cellular layers from 3–4 in the normal rat epidermis to 12–14. The cells in this thickened edge commence to migrate out over the denuded surface and under the eschar. During this initial phase the cytoplasmic R.N.A. is visually unchanged. The 3rd through the 8th day are characterized by continued migration of the epithelium and increased proliferation of its lower layers. During this period, the layers of migrating epithelium exhibit an overall increased concentration of R.N.A. (Fig. 1, Fig. 2). This is especially evident in the basal cells where the R.N.A. is seen densely packed as fine granules about

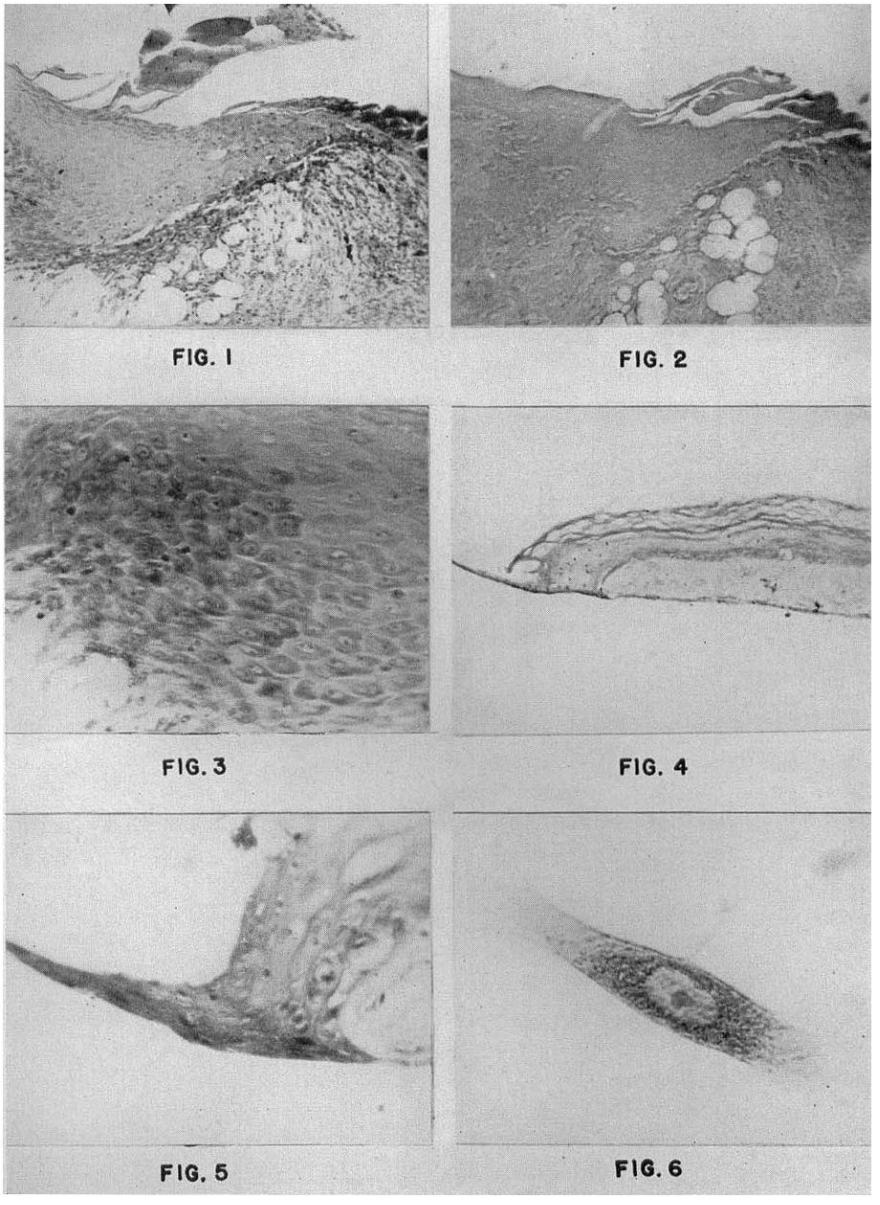

FIG. 1. Edge of a 6 day rat wound, stained with methyl green-pyronine. The epithelial layers rich in R.N.A. as indicated in the photograph by their darker cytoplasm are seen at the right migrating under the eschar. The remaining border epithelium is thickened and the cells of the basal and lower stratum spinosum contain large quantities of cytoplasmic R.N.A. × 100

FIG. 2. Control section of burn in Fig. 1. Prior to staining with methyl green-pyronine the section was digested with ribonuclease. R.N.A. is absent from all layers. × 100

FIG. 3. High power detail of the basal and stratum spinosum layers pictured in Fig. 1. The cytoplasmic R.N.A. appears granular and is grouped about the nuclei. × 430

the nucleus (Fig. 3). After completion of epithelialization the number of layers gradually returned to the 3 or 4 usually found in resting epidermis. At this time the R.N.A. content of the cells is reduced to normal. From the above it is evident that the periods of increased cytoplasmic R.N.A. content are closely related to phases of rapid growth rather than cellular migration. The nucleoli also contain R.N.A. but no change in relative concentration could be detected visually.

2. Thymonucleic acid, found only in the nuclei and stained by the Feulgen technic also presents a gradient pattern. In normal rat skin the most densely stained nuclei occur in the basal and lower stratum spinosum layers. The keratinizing and keratinized superficial layers with their pyknotic nuclei appear less densely stained. Throughout wound healing no visual changes were detectable in the lower layers of normal skin as compared to skin overlying the wound.

Cultured Human Skin

1. Fifteen explants of human skin were cultured on collodion membranes and subsequently sectioned and examined histochemically for ribonucleic acid content. The chain of events occurring in culture are similar to those found in wound healing. The epithelium after an 18–24 hour lag phase migrates down over the dermal edge of the explant and then grows out along the collodion membrane. At the time of culture the human explant epithelium contains an R.N.A. gradient similar to that seen in rat skin (Fig. 4). The migration of epithelial cells that commences 24 hours after culture is accompanied by a rise in their cytoplasmic R.N.A. content (Fig. 5). This increase is also evident in the epithelial cells at the explant edge; the site of maximum growth. The cells at the latter site form a more reliable index of R.N.A. concentration as they are not thinned out or flattened like those in the outwandering area. All cells of the latter area maintain their large quantities of R.N.A. until degeneration commences on the 8th and 10th day. Throughout this period no change in concentration occurs in the explant itself.

Whole mount preparations in which the isolated epithelial cells are spread out on the cover slip surface permit an accurate localization of R.N.A. The distribution is centered about the nucleus and decreases outward from this site

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FIG. 4. Explant of human skin fixed 3 days after culture on a collodion membrane and stained as above. The basophilia resulting from R.N.A. is found chiefly in the cells of the basal and lower stratum spinosum. No increase in R.N.A. content of the outwandering cells is evident while they are still in the migrating phase. × 100.

FIG. 5. Cultured explant of human skin fixed on the 6th day and stained with methyl green-pyronine. Cells in the outwandering epithelium now in the proliferative phase are rich in R.N.A. × 430

FIG. 6. Whole mount of cultured human epithelial cell stained as above. The granular deposition of R.N.A. decreasing in concentration at a distance from the nucleus is visible. A thin tension line extends to a cell not pictured in the photomicrograph. The single nucleolus also stains with the basic dye indicating R.N.A. × 980

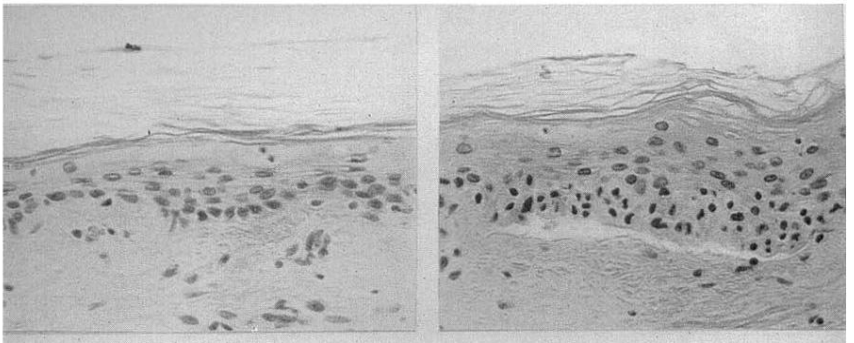

FIG. 7

FIG. 8

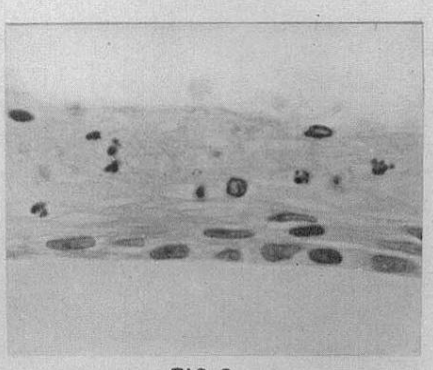

FIG. 9

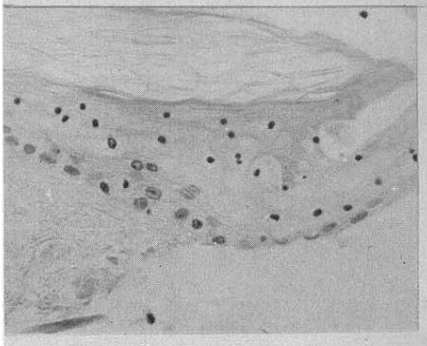

FIG. 10

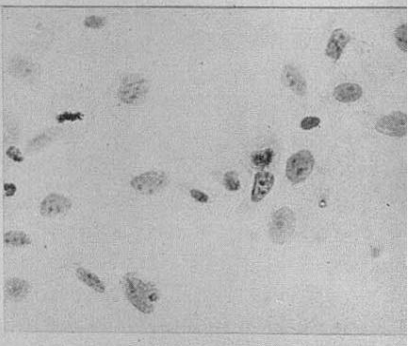

FIG. 11

FIG. 7. Detail view of the dermal-epidermal junction in human skin after 4 days of culture. The section was stained by the Feulgen method and counter stained with light green. The nuclei of the cells in the basal layer appear morphologically normal and are rich in D.N.A. The more superficially located layers present a gradual keratinization accompanied by pyknosis and ultimate loss of D.N.A. × 430

FIG. 8. A different area of the explant in Fig. 7 showing the dermal-epidermal junction and similarly stained. There is a "reversal" of nuclear morphology. The basal and lower stratum spinosum cells are pyknotic, while those in the superficial layer resemble "normal basal cells". × 430

FIG. 9. High power detail of the outwandering epithelium, stained as in Fig. 7. Morphologically this epithelium resembles that of normal skin except for the apparent absence of keratinization. × 980

FIG. 10. Edge of an explant stained as in Fig. 8. The region photographed is that of maximum mitotic activity; one cell in the basal layer is dividing. × 430

FIG. 11. Whole mount of cultured human skin consisting of outwandering cells several layers thick. Preparation stained with the Feulgen technic. The variability in nuclear size and staining is evident. Two cells in mitosis are present. × 430

(Fig. 6). The stained particles are very finely granular and no clumping was found except in the rounded degenerate cell. Cytoplasmic tension lines extending from cell to cell were rich in R.N.A. None was ever found near the border of the "undulating membrane". The nucleoli were basophilic also but did not seem to vary in intensity. The number and size of the nucleoli varied with the activity of the cell itself. Resting cells contained 1 or 2 nucleoli of similar size. Prior to cell division the number frequently increased to 4 or 5. In all degenerate cells, there was a fusion of nucleoli resulting in one large nucleolus.

2. Thymonucleic acid concentration of sectioned explants were examined in 12 cases. At the time of culture, the nuclei in the lower epithelial layers stained heavily. Those in the keratinizing and keratin layers contained less D.N.A. This relationship was always reversed during the period of culture when separation at the dermal-epidermal junction took place. This occurred at various intervals in the explant with approximately 50% of the cases. These areas revealed morphologic changes in the nuclei of the cellular layers (Fig. 7 and Fig. 8). Those of the basal layer became pyknotic, there was disappearance of the nuclei and increased staining of the entire nucleus. The latter is due to condensation rather than increased D.N.A. content (Leuchtenberger (25)). The nuclei in the more superficially located layers that usually have this appearance were instead similar to those normally found in the lower mitotic layers. The nuclei of the outwandering epithelium exhibited the same pattern found in normal skin (Fig.9). Those in the lower layers were rich in D.N.A. and morphologically resembled basal cell nuclei. Pyknosis, shrinkage of nuclear volume and concentration of D.N.A. occurred in the superficial layers.

Sections of cultured explants stained with the Feulgen technic were used to determine sites of epithelial mitosis. The 12 explants studied were completely sectioned (approx. 175 sections per explant). In each case the majority of mitoses were located within the border of the explant itself (Fig. 10). These mitoses which commence after the lag phase are not visible in whole mount preparations. The cells derived at this site appear to be those which form the outwandering epithelium. This is construed from the fact that the borders of the explant epidermis are at no time reduced in thickness or number of layers. Frequently mitoses were seen in the outwandering epithelium. They occurred in a ratio of approximately 1:10 with those at the explant edge. These mitoses were often characterized by an excess number of chromosome indicating polyploidy or else were degenerate. Scattered mitoses also were observed throughout the basal or stratum spinosum layer of the explant.

Whole mounts stained for D.N.A. revealed variances in the nuclear size of cultured epithelial cells (Fig. 11). Cultures fixed at 6 or 8 days in general had larger nuclei than those found on the 2nd or 4th days. The larger nuclei stained less intensely indicating a dilution of the available D.N.A. All cells observed during mitosis contained chromosomal strands heavily stained with D.N.A.

DISCUSSION

It has become clear in recent years that ribonucleic acid plays an important role in protein synthesis, although its precise function remains unknown. Cells

maintaining rapid growth rates contain large quantities in their cytoplasm. In normal skin this is manifested by a decreasing gradient extending from the basal cells to the stratum corneum. This gradient can be greatly increased when growth is stimulated. Biesele (2) has shown that within 12 hours a rise in R.N.A. occurs in mouse skin made hyperplastic by treatment with methylcholanthrene. A similar change is frequently seen in tumors (Stowell (36)). During wound healing epithelium also undergoes a phase of rapid growth. Prior to this however a migration over the denuded surface takes place. This period is not characterized by any change in cytoplasmic R.N.A. content. With the onset of growth in the lower layers there is a concomitant rise in the quantity of this substance. Autografts of human skin have recently been shown to exhibit a similar phenomenon (Scothorne (31)). These findings in wound healing agree completely with the present concepts relating R.N.A. to protein synthesis.

Epithelium cultured *in vitro* undergoes essentially the same phases described above for wound healing. During the initial migration there is no change in the cytoplasmic R.N.A. content—the subsequent rapid growth limited to the cells at the border of the explant and in the lower layers of the outwandering epithelium reflected a similar increase in R.N.A. This relationship between growth and ribonucleic acid content in culture has also been biochemically determined in fibroblasts (Davidson et al. (16)). Unlike fibroblasts however, cultures of epithelium possess cells both capable and incapable of division. The increase in cytoplasmic R.N.A. content is selectively restricted to those cells in which proliferation occurs. This is good support against arguments that a diffusion from the culture media is the source of additional R.N.A. Furthermore it has also been found that no relationship exists between the nucleic acid concentration of embryonic tissue and the growth promoting characteristics of its extracts (Davidson et al. (13)). Accumulated evidence indicates that synthesis of nucleoproteins by cultured cells is a necessary prerequisite to cell division (Davidson et al. (14)). The materials for such synthesis are readily available in the embryo extract and plasma of the culture media. The elevated ribonucleic acid content in proliferating epithelial cells both *in vitro* and *in vivo* is another striking parallelism between their metabolism under these different conditions.

Recently Hsu (22) attempted to determine the precise nature of the epithelial outgrowth found in culture. Mitotic counts of whole mounts revealed that only 32 slides of 240 examined presented evidence of cellular division. These for the most part were abnormal with degeneration after metaphase or anomalous anaphases. He concluded that skin cultured by present methods (Lewis et al. (24)) resulted in survival cultures and that lack of normal mitosis indicated cellular malnutrition.

The use of sectioned material made feasible by culturing on collodion membranes offers an opportunity to re-examine this problem. In these sections it is evident that at no time is there an appreciable diminishing in the thickness of the explant epithelium. This precludes the possibility of cellular migration from the explant epithelium as the source of the outwandering cells. Where then do the outwandering cells come from? During *in vivo* wound healing, following a lag phase of 4–5 days the mitotic activity increases to twice that of normal

(Blumenfeld (4)). These mitoses occur for the most part at the edges of the wound (Loeb (27), Hartwell (21), Arey (1), and Bishop (33)). Sections of rat wounds have confirmed this. A similar situation has been found to exist in cultured skin, the majority of mitosis occurring in the basal and stratum granulosum layer within the borders of the explant. These divisions which appear normal and are the source of the cells in the epithelial out growth are not visible on whole mount examination. Mitosis also occur to a lesser extent within the lower layer of the explant and in the outgrowth itself. The reason for abnormal divisions at this latter site is not known. There appears to be no diminishing in the nuclear thymonucleic acid content. It is known that D.N.A. content is relatively stable regardless of the status of cellular nutrition. Davidson (15) has shown that in fasting states the R.N.A. of liver cells decreases but the D.N.A. remains constant. Work with isotopes (Furst et al. (19)) has reemphasized this stability. Evidence of poor nutrition during culture has not been obtained in relation to glycogen metabolism (Washburn (37)) or nucleic acid metabolism. In both cases the outwandering cells exhibit similar patterns to those seen *in vivo*. One fact which may yield a clue is that outwandering epithelium lacks a basement membrane and underlying dermis. It is evident on sectioned material that areas of separation at the dermo-epidermal junction do occur in the explant. At these areas the epithelium loses its normal morphological appearance. The cells in the lower layer become pyknotic and do not divide. The possibility that contact with the dermis is necessary for normal epithelial growth cannot be excluded.

A gradual increase in nuclear size during culture was observed by Hsu (22). Whole mounts stained with the Feulgen technic show considerable individual variation at any one time. The larger nuclei stain less intensely indicating that increase in size is not accompanied by an increase in thymonucleic acid. A similar phenomenon has been found in skin treated with methylcholanthrene (Pullinger (30), Cowdry and Paletta (12), and Kraemer (23)). It has been concluded that this is in part due to polyploidy (Biesele (2)). Cowdry et al. (12) followed the nuclear viscosity of methylcholanthrene-treated epithelium and found it lower than in normal skin. They concluded that an increased water content was a contributing factor.

SUMMARY AND CONCLUSIONS

During wound healing, epidermis undergoes first a phase of cellular migration followed by one of growth. In rats, the latter phase is characterized by an increased concentration of cytoplasmic ribonucleic acid. This is restricted to those cells where mitosis occurs, namely in the basal and lower stratum spinosum layers. A similar response is found in cultured human epithelium also being limited to those layers where proliferation occurs.

During culture, explants frequently reveal areas of separation at the dermo-epidermal junction. Epithelium overlying these areas is morphologically abnormal with degeneration in the lower layers and preservation of a "basal cell appearance" in the more superficial layers.

A close similarity exists between epithelium migrating over denuded burn

surfaces and the outwandering cells found in culture. In both cases the initial migration is followed by intense proliferation just within the borders either of the surrounding normal skin (in burns) or of the explant (in culture). Mitosis occurs to a lesser extent in the outwandering epithelium during culture, but a large percentage of these are abnormal. Thymonucleic acid concentration of the nuclei either in burns or culture does not appear to vary appreciably.

The ribonucleic and thymonucleic acid metabolism of epithelial cells during *in vivo* wound healing and *in vitro* cultures appears identical.

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